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Award Number: W81XWH-11-1-0494

TITLE: The Snail-Induced Sulfonation Pathway in Breast Cancer Metastasis

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REPORT DATE: July 2013

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2013	Annual	1 July 2012 - 30 June 2013

4. TITLE AND SUBTITLE

The Snail-induced sulfonation pathway in breast cancer metastasis

5a. CONTRACT NUMBER
W81XWH-11-1-0494

5b. GRANT NUMBER
W81XWH-11-1-0494

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Wistar Institute
Philadelphia, PA 19104-4265

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project seeks to establish a key role for cellular sulfonation enzymes in the metastatic progression of breast cancer. To this end we have accomplished the following tasks in this second year of support:

1. Further characterization of Tetracycline inducible WT Snail and mutant Snail vectors and cell lines.
2. Demonstration that the PAPSS2 knockdown vectors do not work and/or are very leaky and must be further developed.
3. Demonstration MCF-10A cells undergo dramatic morphologic EMT in the presence of WT Snail expression, with the downregulation of the Cell adhesion molecule E-cadherin and the upregulation of the mesenchymal markers vimentin and fibronectin.
4. Characterization of antibodies to PAPSS1 and PAPSS2 enzymes and demonstration that they cross react with one another making their use in IHC very problematic.
5. Request for, and granting of an extension of this research project in order to solve the unexpected technical problems.

15. SUBJECT TERMS
Snail, Metastasis, PAPSS2, Sulfonation, EMT

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	7	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:

Background: The Snail transcription factor is a strong inducer of epithelial-mesenchymal transition (EMT) in normal embryonic development and tumor metastasis. In mice, Snail is spontaneously up-regulated during tumor recurrence in the mammary gland and high Snail expression strongly predicts decreased relapse-free survival in women with breast cancer, suggesting that Snail may play a critical role in tumor recurrence. All evidence indicates that Snail induces EMT and metastasis at least partially due to repression of *E-cadherin* and other genes encoding cell junction proteins, thereby altering cell adhesion. Thus, previous studies have focused on defining Snail-associated repression complexes and identifying genes repressed by Snail. Interestingly, our preliminary studies provided with the original proposal demonstrated that expression of the *PAPSS2* gene, which encodes the enzyme 3 -phosphoadenosinse 5-phosphosulfate synthase 2, a rate-limiting component of the sulfonaiton pathway, was induced by Snail in breast cancer cells. Depletion of PAPSS2 expression in highly metastatic breast cancer cells led to acquisition of the epithelial phenotype. PAPSS2 catalyzes the synthesis of 3 phosphoadenosinse 5-phosphosulfate (PAPS), which serves as the universal sulfonate donor for all cellular sulfonation reactions: a process of the transfer of a sulfonate group (SO3-1) from PAPS to appropriate acceptor molecules.

Hypothesis/Objective: We are examining the hypothesis that protein sulfonation induced by Snail is required for the induction of EMT and metastasis in breast cancer.

Specific Aims: We are performing experiments to: 1) test the hypothesis that PAPSS2 is required for maintaining the mesenchymal phenotype and invasive property of the breast cancer cells and 2) test the hypothesis that Snail-stimulated sulfonation of proteoglycans (PGs) are crucial for the induction of EMT and metastasis. **These experiments were approved in the original Proposal and Statement of Work and our experimental course has not deviated from this plan. Details of our progress are presented below.**

BODY:

We have made limited progress in the second year of support for this project primarily due to un-forseen technical difficulties. A key goal was to establish cell system wherein the expression and function of PAPSS2 and overall cellular sulfonation could be measured and correlated with both Snail levels/activity and correlate with metastatic potential of the cells. Our original goal was to characterize this in many established cell lines with already well-defined Snail levels and metastatic potential. Over the course of the year, this strategy has proved problematic as the phenotypes and the expression levels in these well established, non-clonal cell lines are quite variable. In order to circumvent these problems, we have elected to jump to the strategy described in Aim 2 and derive clonal engineered cells lines using inducible Snail expression vectors. This has been well accomplished but has also significant, completely unforeseen problems associated with it. This was documented in last years progress report but briefly summarized here:

A primary goal of this aim was to establish and inducible and reversible system to effect snail-mediated EMT in breast cancer cell lines using inducible plasmid or viral vectors and look at the induction on PAPSS2. This was critical in order to map the epigenetic marks placed in chromatin during both the induction and reversion of the EMT differentiation event and the role of sulfonation in these processes. We proposed to do this in the original grant proposal by making Snail fusion proteins to the Estrogen receptor hormone binding domain (HBD-TM) thus creating a tamoxifen responsive protein which could be turned on and off. Unfortunately, despite tremendous effort over the past 2 years, we have found that this system does not work with Snail family zinc finger proteins;

The Snail-HBD fusions were poorly expressed in cells, the inductions were leaky and often unresponsive to hormone, and the regulation of both a Snail target gene and the EMT phenotype was variable and overall unsatisfactory. Thus about 1 year ago we moved to an entirely different inducible vector system, PLUT. This is based upon a TET-ON lentiviral system developed by my previous post-doctoral fellow Alexey Ivanov. He created a series of lentiviral vectors, which encode the TET–ON repressor, and a separate insert site for a candidate cDNA under the control of TET operators. The beauty of this system is that it is completely encoded in a single lentiviral vector, and thus separate TET-ON encoding vectors are not required. We created wt and mutant Snail vectors in this system, harvested high titer lentiviral stocks and used these to infect a myriad of breast cancer cells. Clonal derivatives form these cultures were isolated expanded and tested for induction of Snail protein by Doxycycline treatment, and for induction of the EMT phenotype. In the past year we have isolated and tested literally hundreds of clonal lines from separate transfections in order to identify novel founder lines. We thus now have beautiful set of stably transfected cell clones that have a low background level, but that are robustly inducible for Snail after Dox treatment. An example is shown below (Fig 1) in both MCF10A cells and murine 67NR cells: Both wt Snail and mutant TP-AA Snail is robustly induced by Dox treatment of 67NR cells

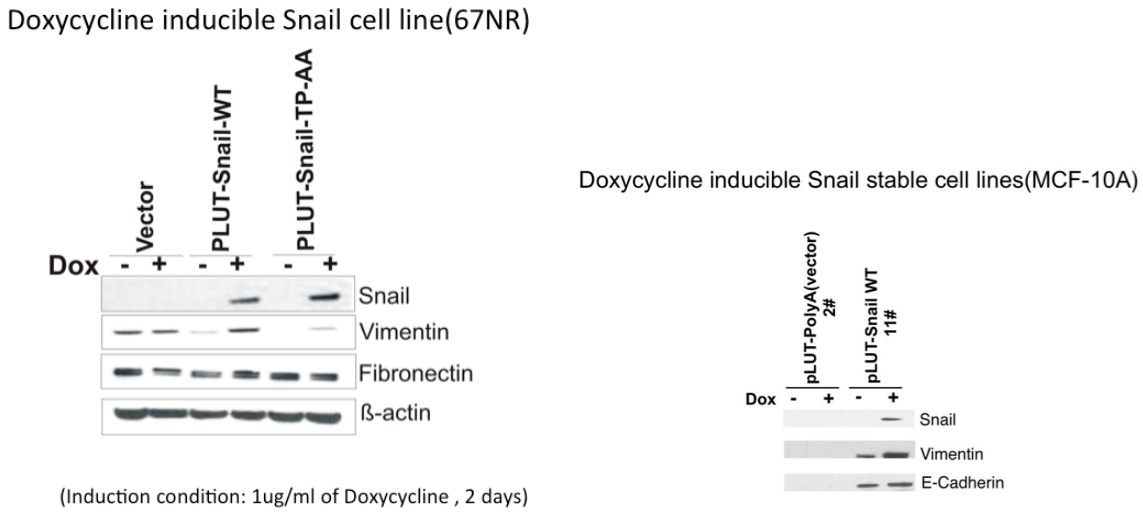

Figure 1: Creation of Doxcycline Inducible Snail Clonal Cell lines from MCF10A and 67NR cells.
Upper left, Western blot of Snail and other EMT markers in two clonal cell lines expressing either wt Snail or the Mutant TP-AA which destroys the T177 Phosphorylation site

A second major goal of the second year was to generate and characterize highly specific shRNA knockdown vectors that specifically target each PAP enzyme isoform. We have done so by evaluating a number of different vector designs and nucleotide sequences. In transient transfectins, these vectors have been shown to specifically target either one or the other isoforms. These vectors have now bee introduced into the cell lines shown above and stably knockdown clones are under selection. These clones will be analyzed for 1) knockdown of each enzyme by Western blot, 2) Influence on cell growth and motility, 3) effect on the metastatic phenotype. All of these experiments were in the original plan of the proposal and were scheduled to be accomplished in Year 2.

However, we have had unexpected experimental approach difficulties which were compete unforeseen during design of the original project. Specifically, a cornerstone experimental procedure for testing our hypothesis that PAPSS2 controls the mesenchymal phenotype during is the ability to stably eradicate expression of PAPSS2 gene expression in various cell lines and in tumors. The state-of-the-art technique for doing this is either miRNA mediated gene knockdown. However, this simply does not work in our hands for this particular gene. We have tried multiple independent experimental approaches to miRNA KO including transient transfection, (either naked RNA or

synthetic oligos), transient vector (lentiviral-mediated) expression of miRNA , and finally selection of independent clonal lines of stable expressing miRNA vectors. The results show that contrary to any gene which we have ever worked with, none of these techniques are able to silence PAPSS2 (or PAPSS1). The cells either immediate shut off expression of the transgene miRNA vector or the cells die. Thus either PAPSS2 is an extremely important gene for normal cell homeostasis, or there are very significant off-target effects in our constructs. However the latter explanation is very curious as we have used multiple independent targeting sequences for PAPSS2 which should control for for these effects. In order to perform the comparative genomic and proteomic analyses of metastatic cell which do or do not have PAAPSS2 expression as originally proposed, we must be able to do this. We will now utilize a vector system worked out in this laboratory and just now coming online. It involves using tightly controlled, inducible vectors for miRNA expression. This has worked in ours and others hands for KO of recalcitrant target genes such as PAPSS2.

Thus we successfully request an extension from the DOD to perform these critical experiments in fulfillment of the original AIMS. To be sure, this was not a request to change the original specific aims, simply a request to extend the experimental approach into an area that we did not think we would have to.

Other than the problems fully described above, there are no deviations of research strategy to report at this time.

KEY RESEARCH ACCOMPLISHMENTS: YEAR 2

This project seeks to establish a key role for cellular sulfonation enzymes in the metastatic progression of breast cancer. To this end we have accomplished the following tasks in this second year of support:

1. Further characterization of Tetracycline inducible WT Snail and mutant Snail vectors and cell lines.
2. Demonstration that the PAPSS2 knockdown vectors do not work and/or are very leaky and must be further developed.
3. Demonstration MCF-10A cells undergo dramatic morphologic EMT in the presence of WT Snail expression, with the downregulation of the Cell adhesion molecule E-cadherin and the upregulation of the mesenchymal markers vimentin and fibronectin.
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5. Request for, and granting of an extension of this research project in order to solve the unexpected technical problems.

REPORTABLE OUTCOMES: None

CONCLUSION: In summary we are highly motivated to describe this completely novel cellular sulfonation pathway controlled by Snail through activation of gene expression to regulate EMT and metastasis in breast cancer cells. Our preliminary studies have shown that Snail induces the key enzyme in the sulfonation pathway called phosphoadenosinse phosphosulfate synthase 2 (PAPSS2) in breast cancer cells. PAPSS2 catalyzes the synthesis of PAPS, the only sulfate donor for all sulfonation reaction in cells. Strikingly, inactivation of this enzyme in the highly aggressive, mesenchymal-like, metastatic human breast cancer cells leads to profound acquisition of the epithelial phenotype, suggesting a Snail stimulated posttranslational modification of proteins and

other biomolecules is critical in the regulation of EMT and metastasis. However, virtually nothing is known of this sulfonation pathway in the context of the transcriptome regulated by Snail in tumors. The next year of research funded by this grant should produce some striking discoveries in this pathway. We are implementing significant changes in strategy and experimental approach in order to rectify problems with addressing the question.

REFERENCES: None

APPENDICES: None